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Role of CD4+CD25+ Regulatory T Lymphocytes in Experimental Toxoplasmosis

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ROLE OF CD4+CD25+ REGULATORY T LYMPHOCYTES IN EXPERIMENTAL TOXOPLASMOSIS

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IN EXPERIMENTAL TOXOPLASMOSIS

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Toxoplasmosis is an important cause of congenital disease, and it is one of the most common opportunistic infections in patients with acquired immunodeficiency syndrome. The need for a reliable experimental model of this infection is crucial not only for achieving a better understanding of the patho-physiology of the disease, but also for developing better methods for evaluating new therapeutic regimens. The purpose of the present study was to investigate the role of CD4+CD25+ T regulatory lymphocytes in mice infected with *Toxoplasma gondii*. T regulatory (Treg) cells have been shown to play an important role in our immune system in controlling the activity of other T lymphocytes. These cells are differentiated from other T lymphocyte populations based on the co-expression of CD4 and CD25 and expression of the Foxp3 gene. The results of several recent studies have suggested that certain pathogens may be able to increase their survival in the host by exploiting T reg cell activity. T regulatory cells have been shown to control the persistence of the protozoan parasite, *Leishmania major*, in mice; however, this population of cells plays only a limited role during murine infection with *Trypanosoma cruzi*. In the present study we have investigated the role of Treg cells

during murine infection with the ME49 strain of *T. gondii*. In vivo depletion of Treg cells was accomplished by injecting mice with a monoclonal antibody (Mab) isolated from the 7D4 rat hybridoma cell line. This Mab is specific for the interleukin-2 receptor chain (also known as CD25). Female Swiss Webster mice of approximately 6-7 weeks of age were depleted of Treg cells by intraperitoneal injection of 400µg of Mab, mice were injected once 7days prior to infection, and a second time 1 day prior to infection, with 20 tissue cysts of *T. gondii*. Mouse weight and tissue cyst numbers were monitored to evaluate the impact of Treg depletion on the outcome of infection. Our results suggest that depletion of Treg cells has little measurable impact during the acute stage of infection with the ME49 strain of *T. gondii*. Further studies will be required to determine what role, if any, these cells play in the chronic stage of murine toxoplasmosis.

INTRODUCTION

Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular protozoan parasite responsible for the disease known as toxoplasmosis. *Toxoplasma gondii* infects most warm-blooded animals including humans. The definitive host of this single-celled parasite, and the host in which the sexual cycle of the parasite takes place, is the cat. Nicolle and Manceaux first described the organism in 1908 after they observed the parasites in the blood, spleen, and liver of a North African rodent, *Ctenodactylus gondii* (cited in Black et al., 2000). *Toxoplasma gondii* infects a large proportion of the world's population but uncommonly causes clinically significant pathology. However, certain individuals are at high risk for severe or life-threatening disease due to this parasite. Individuals at greatest risk include fetuses, newborns, and immunologically impaired patients. Toxoplasmosis has great economic importance in veterinary medicine because it causes abortion in cattle and significant neonatal loss. *Toxoplasma gondii* infection is widespread in humans, and in the United States and United Kingdom, the infection rate is estimated to be 16-40% of the population. In Central and South America and continental Europe, the infection rate is estimated to be 50-80% (Hill and Dubey, 2002).

Most infections with *T. gondii* in humans are asymptomatic and infection may be congenitally or postnatally acquired. Approximately 10-20% of those with an acute infection will have enlarged lymph nodes in the cervical and inguinal region as well as flu-like symptoms (fever, headache, muscle pain). The infection is generally self-limited and the symptoms usually resolve in a few months. Toxoplasmosis has a variable outcome depending on the interaction of many factors, including the route of infection,

the host immune system, and host genetics. Human infections are generally acquired by ingestion of undercooked meat or raw meat containing tissue cysts or from the ingestion of oocysts (Hill and Dubey, 2002). Encephalitis is an important and potentially fatal manifestation of toxoplasmosis in immune-compromised patients including patients with AIDS. Symptoms may include headache, disorientation, drowsiness, hemiparesis, reflex changes, and convulsions. Coma and death also occur frequently (Hill and Dubey, 2002). Toxoplasmic encephalitis frequently occurs in AIDS patients following reactivation of a latent infection due to the long term presence of the tissue cyst stages of the parasite.

Acute toxoplasmasis acquired during pregnancy may result in the death of the fetus or in serious congenital complications such as blindness, deafness or central nervous system disorders (Switaj et al., 2005). Thus, the importance of the organism as a pathogen of humans and domestic animals has inspired a significant amount of research in recent years.

Biology, Life Cycle, and Transmission of *T. gondii*

Intracellular stages of *T. gondii in* the vertebrate host are found in many types of cells and tissues, including muscle tissue, brain tissue, and intestinal epithelium. Members of the family Felidae serve as definitive hosts for *T. gondii*, and a wide variety of birds and mammals serve as intermediate hosts (McCarthy and Davis, 2003). Toxoplasmosis is transmitted to humans generally by the ingestion of tissue cysts in infected meat or by the ingestion of oocyst stages present in food contaminated with cat feces. Bradyzoites released from the tissue cysts or sporozoite stages released from oocysts penetrate intestinal epithelial cells and begin to multiply. The process of host cell invasion is facilitated by apical organelles which are unique to organisms in the

phylum “Apicomplexa” (Kim and Weiss, 2004). Studies of *T. gondii* have revealed that host cell invasion is a carefully organized process that is accompanied by the sequential release of micronemes, rhoptries and also by dense granules. Within the host cell, the parasite resides within a specialized vacuole termed a “parasitophorous vacuole” that forms from the plasma membrane during invasion, but remains segregated from other endocytic compartments. This parasitophorous vacuole does not acidify or fuse with the host lysosomal system (Shaw et al., 1998). *Toxoplasma gondii* shares many features with other Apicomplexan parasites but is unusual in its extremely broad host and tissue specificity. The parasite exhibits typical 'zoite' morphology, its highly polar structure being dictated by the complex cytoskeleton. Molecules on the surface of the zoite are prime candidates for interaction with the host cell (Smith, 1993).

The life cycle of the parasite is divided between the feline and non-feline infections, which are correlated with the sexual and asexual phases of replication respectively (Black and Boothroyd, 2000). The sexual stages of the parasite occur within the intestinal enterocytes of Felids. After ingestion of the tissue cysts, liberated parasites invade the enterocytes, undergo several rounds of division and differentiate into micro & macro gametocytes. The gametocytes fuse to form a zygote or “oocyst” that is shed into the environment with the cat’s feces. The oocyst undergoes meiosis, producing an octet of highly infectious “sporozoites” that are resistant to environmental damage and may persist for years in a moist environment (Denkers and Gazzinelli, 1998). Soil and plants contaminated with oocysts are the most common sources of infection for herbivorous intermediate hosts such as cattle, sheep, and rodents.

In the intermediate host, *T. gondii* undergoes an asexual phase in its life cycle that includes rapidly multiplying tachyzoites as well as slowly multiplying bradyzoites (Dubey and Beattie, 1988). The rapid replication and dissemination of tachyzoite stages in the intermediate host is responsible for the acute phase of the disease. Bradyzoites (characterized by their low metabolic activity and slow rate of replication) remain in the tissues within tissue cysts which appear 7 to 10 days post infection. These tissue cysts are found predominantly in the central nervous system and in the muscle tissues where they may reside for the life of the host (Black and Boothroyd, 2000). The development of the tissue cysts throughout the body signals the chronic stage of the infection.

Tachyzoites are approximately 5μm long and replicate inside the cell with a generation time of 6-8 hrs (Black and Boothroyd, 2000). When the host cell becomes packed with tachyzoites, the host cell plasma membrane ruptures and the parasites are released into the extra-cellular milieu. The free tachyzoites can then infect any nucleated cell and continue their intracellular replication. These tachyzoite stages are highly virulent if they are not controlled by the host immune system (Dubey et al., 1998). Generalized toxoplasmosisis due to the uncontrolled replication of tachyzoite stages is always fatal (Denkers and Gazzinelli, 1998). Indeed, many studies show that normally avirulent strains of *T. gondii* are highly virulent in T-lymphocyte deficient animals (Gazzinelli et al., 1991). In immuno-competent hosts, the immune response intensifies, the reproduction of the parasite slows and numerous bradyzoites develop inside the cells of the nervous system, heart, and muscles.

Transmission of *T. gondii* also occurs through blood transfusions and organ transplants. Tissue cysts within the transplanted tissues of the donor or in the latently

infected transplant patient are the most likely source of the infection (Hill and Dubey, 2002).

Immunity & Pathogenesis

During the acute phase of infection with *T. gondii*, the proliferation of tachyzoite stages results in the death of the involved host cell and the stimulation of a mononuclear inflammatory reaction (Plorde, 1990). The parasite may then spread to local organs and to distant organs by the lymphatic system and bloodstream. The most common symptoms of acute toxoplasmosis are painful, swollen lymph glands, fever, headache, muscle pain, anemia, and possible lung complications. In immune-deficient hosts, rapid multiplication of the tachyzoites results in necrosis in the intestinal and mesenteric lymph nodes. Focal areas of necrosis may then develop in many other organs. In normal immune-competent hosts, *T. gondii* infection is controlled by a strong cell-mediated immune response that shuts down replication of tachyzoites and leads to the formation of dormant tissue cysts (Roberts and Janovy, 1996). Cell mediated immunity is thought to be the major host factor preventing the reaction of chronic infection as well as determining the acquired resistance to re-infection (Gazzenelli et al., 1991). Both CD4+ and CD8+ T cells responses are involved in the resolution of the infection (Khan et al., 1994). Natural killer (NK) cells also appear to play a critical role during *T. gondii* infection due to the production of interferon gamma (IFN-γ), which in turn activates macrophages into a microbicidal state. Many studies have implicated IFN- γ as a major cytokine involved in the immunological control of acute infection and the prevention of reactivation of latent infection (Araujo, 1992).

The cytokines IL-2 and IL-12 also appear to be principal mediators of immunological resistance during *T. gondii* infection (Khan et al, 1994). IL-2 is required for priming and maintaining a Th1 controlled cell-mediated host response, and IL-12 is an essential cytokine that stimulates the proliferation of NK cells and CD8+ T cells (Araujo, 1992). Chronic infection results when immunity builds up sufficiently to depress tachyzoite proliferation and stimulate the formation of tissues cysts. Chronic active or relapsing infection within retinal cells by tachyzoites can cause blind spots and extensive infections of the central macular area, which may lead to blindness (Roberts and Janovy, 1996).

Congenital infection with *T. gondii* occurs through the acute infection of the expectant mother. The severity of congenital infection depends on the stage of the pregnancy at the time of acute infection (Black and Boothroyd, 2000). Spontaneous abortions and congenital neurological disorders such as blindness and mental retardation commonly occur. Congenital toxoplasmosis is also a frequent cause of intracerebral focal lesions resulting in toxoplasmic encephalitis (Wong and Remington, 1993).

T regulatory cells

Our immune system generally protects us from an infinite number of potentially pathogenic microorganisms while avoiding the constituents of our own body. This phenomenon is known as “self tolerance”. Failure of immunologic self tolerance often leads to the development of “autoimmune disease” (Sakaguchi, 2000). The thymus plays an important role in the maintenance of self tolerance, due to its role in the clonal deletion of self reactive T cells however, additional mechanisms including the induction

of T cell anergy, and "immunological ignorance" are believed to be necessary for the maintenance of peripheral self-tolerance (Zinkernagel, 1997).

The existence of lymphocyte populations that suppress adaptive immune responses (both antibody-mediated and cell mediated) has been proposed by many different investigators since the early 1970's. T cells capable of suppressing the development of autoimmune diseases –so-called "suppressor T cells" - were first proposed by Gershon and Kondo in 1971. Suppressor T cells were thought to be a specialized population, whose effects were mediated by secreted antigen specific factors. It was suggested that these suppressor T cells restricted the induction or expression of effector T cells and thereby prevented or terminated exaggerated responses or, in the case of self-reactive T cells, autoimmune disease (Garra and Vieira, 2004). These suppressor T cells were believed to represent a crucial element in the regulatory network responsible for controlling immune responses and maintaining peripheral tolerance (Mittrucker et al., 2004). Following multiple failed attempts to elucidate the molecular basis for T suppressor cell activity, the concept of the T suppressor cell fell rapidly out of favor, and the term disappeared from the immunological literature for many years.

In 1995, Sakaguchi and his associates described a population of naturally occurring CD4+ CD25+ T cells in mice that constituted approximately 5-10% of all T helper (Th) cells. These CD4+ CD25+ T cells were shown to exhibit potent regulatory functions both *in vitro* and *in vivo* (Sakaguchi et al., 1995; Sakaguchi et al., 2001). These "suppressor T cells", now less provocatively renamed "regulatory" T cells (Treg cells), have been isolated from both mice and humans, and their suppressive or regulatory capacity has been convincingly demonstrated. Although it is clear that the clonal deletion

of self-reactive T cells in the thymus is responsible for central tolerance, the presence of auto-reactive T cells in normal healthy individuals indicates that potentially pathogenic, self-reactive T cells form a part of the normal T cell repertoire (Fowell and Mason, 1993).

Development of naturally occurring CD4+CD25+ regulatory T cells

The thymus has been shown to be an important organ in the generation of Treg cells. Papiernik et al. (1997) were the first to demonstrate the presence of CD25+ T cells in the thymus. In their studies, the expression of CD25 appeared to be induced at the CD4+ single positive stage in both humans and in rodents (Beissert et al, 2006). These Treg cells were shown to inhibit T-cell proliferation and the development of inflammatory bowel disease as well as diabetes in mice (Sakaguchi et al., 2001). Therefore, an important role for these cells in the prevention of autoimmunity was demonstrated.

The results of grafting studies revealed that the thymic epithelium is involved in the differentiation of Treg cells. Grafting of allogeneic thymic epithelium induced tolerance to a variety of peripheral tissues of donor origin, and this tolerance was shown to be mediated by CD4+ T cells (Maloy and Powrie, 2001). Thymic cortical epithelial cells (positive for Class I and Class II major histocompatibility complex proteins) appear to play a key role in the positive selection of CD4+CD25+ T cells (Bensinger et al., 2001). Fluorochrome labeling studies showed that CD25+ cells migrate from thymus to populate secondary lymphoid organs and tissues.

The identification of CD25+ T cells in the CD4+CD8- peripheral T cell pool gave rise to the hypothesis that these cells might interact with thymic medullary Dendritic Cells (DCs) during the process of negative selection (Shevach, 2001). Thymic elements resistant to radiation have been shown to mediate negative selection of the CD4+CD25+ cells within the thymus. CD25 expression is acquired relatively late in thymocyte development, as the cell progresses from the CD4+CD8+ double positive stage to the CD4+CD8- stage. This observation prompted the suggestion that those thymocytes that develop into CD4+CD25+ cells may express a T cell receptor(TCR) with a relatively high affinity for self-peptides presented in the thymus (Jordan, 2001). In this scheme, Treg cell development in the thymus would be directed by relatively high avidity interactions between the TCR and self peptide ligands expressed on thymic Antigen Presenting Cells (APCs). Lower avidity interactions would predominantly promote the development of conventional CD4+CD25- thymocytes. Higher avidity interactions would lead to clonal deletion, and the Treg cells which have lower avidity would represent only a small proportion of positively selected thymocytes, perhaps just enough to maintain self-tolerance in the periphery. Therefore, those T cells in the peripheral repertoire with the highest intrinsic affinity for self- peptide/major histocompatibility complex (MHC) would be the Treg cells (Ramsdel, 2003).

In humans, Treg cells have been found in the thymus, in peripheral blood, in lymphoid organs (tonsils and spleen), and in umbilical venous blood. The human studies carried out so far show many similarities to murine *in vitro* studies, indicating that similar mechanisms are involved in both man and mice.

Phenotypic and functional characterization of CD4+ CD25+ regulatory T cells

Naturally occurring Treg cells represent a subset of CD4+ T cells that meture in thymus. They are characterized by the constitutive expression of CD25 and expression of the transcription factor called Forkhead box p3 (Foxp3), which is a member of forkhead/winged-helix family of proteins.

The expression of FOXp3 appears to be a unique and essential characteristic of naturally occurring Treg cells (Brunkow et al., 2001). Both mice and humans deficient in functional Foxp3 protein suffer from severe autoimmune disease symptoms (Ramsdell, 2003). In addition, analysis of the genetic defects in patients and mice suffering from similar X-linked recessive autoimmune and inflammatory disorders, revealed mutations in Foxp3 (Wildin, 2001). Many other transcription factors also play an important role in the development and lineage commitment of T cells. T-bet has been shown to control the Th type 1 (Th1) cells, and GATA-3 controls the Th type 2 (Th2) cells (Finotto and Glimcher, 2004).

A classic characteristic of CD4+ CD25+ T cells is their lack of a proliferative response following Tcell receptor (TcR) activation or stimulation with mitogenic antibodies. However, it is clear from murine studies that CD4+CD25+ T cells must be activated via their TCR to exert their inhibitory function. A variety of studies have suggested that CD4+CD25+ T cells play a role in the suppression of responses to viral,

bacterial, and protozoal infections (Sakaguchi, 2003). Additionally, CD4+CD25+ T cells have been shown to suppress protective anti-tumor immunity (Wang et al., 2004). In contrast, blocking Treg function with a neutralizing antibody directed against the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induced an increased protective immune response (Loser et al., 2005).

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a protein that is preferentially expressed by Treg cells. Optimal T cell proliferation and acquisition of effector functions requires intracellular signals elicited by both the T cell receptor and by a co-receptor, CD28 (Carreno and Collins, 2002). Although CTLA- 4 binds to the same ligands as CD28 (with much higher affinity), its activation leads to T cell anergy (inactivation). CD28 and CTLA-4 also differ in their expression on different subsets of T cells. CD28 is expressed on most CD4+ T cells and almost all CD8+ T cells, whereas CTLA-4 expression is induced in all T cells transiently after T cell receptor activation. However, among non-activated (resting) CD4+ T cells, CTLA-4 is constitutively expressed on T cells that express Foxp3 (Ziegler, 2006).

A number of caveats preclude the use of CD25 as a unique marker of T reg cells. CD25 is transiently expressed upon the activation of murine Th cells, and its expression is very dynamic (Annacker et al., 2001). The CD4+CD25+ T cell population appears to be heterogeneous, and it is assumed that only a portion of this uncharacterized population of cells exerts regulatory/ suppressive activity (i.e. CD4+ CD25+high cells) (Jason et al., 2003).

Immune regulation by Treg cells

The suppression or regulation of immune responses by T cells occurs by various pathways. If non-professional APCs present antigens to T cells and there is an absence or reduced expression of co-stimulatory molecules such as CD80 or CD86 (also known as B7.1 and B7.2), the result is deficient immune activation and anergy. Anergy is also induced if CTLA-4 is expressed by T cells at the time of antigen presentation. The CTLA-4 glycoprotein interacts with either CD80 or CD86 on dendritic cells and results in the production of indoleamine 2, 3-dioxygenase (IDO). The IDO metabolizes tryptophan and leads to a decrease in the activation of T cells (Maggi et al., 2005; Romagnani, 2006; von Boehmer, 2005).

The interaction of Fas and Fas-ligand (FasL) has been shown to induce the apoptosis of FasL+ T cells. The binding of Fas to FasL leads to the activation of caspase enzymes which results in the degradation of chromosomal DNA and apoptotic cell death (Romagnani, 2006). Stimulation of CD4+CD25+Foxp3+ cells via CD3 and CD46 up-regulates the expression of granzyme A, allowing Tregs to induce the apoptosis of CD4+ and CD8+T cells by perforin-granzyme mediated activation of the caspase pathway (Romagnani, 2006).

The suppressive function of Treg cells is reported to be enhanced in the presence of low interleukin 2 (IL-2) concentrations. This is explained by the fact that Treg cells have a higher level of IL-2 receptor expression; thereby possessing a greater ability to utilize the cytokine and thus deprive other T cells access to IL-2. The cytokine known as IL-2 is a T cell growth factor that is essential for T cell proliferation. Its deficiency

results in hampered T cell multiplication and activation. This was postulated to be an important mechanism of Treg suppression that is independent of cytokines such as IL-10 and TGF-β (van Oosterhout and Bloksma, 2005; Yamazaki et al., 2006; Chatila, 2005).

In patients with atopic dermatitis, there is a remarkable absence of CD4+CD25+Foxp3+ Treg cells within skin lesions, and a correlated lack of effector T cell suppression (Verhagen et al., 2006). One of the major cytokines produced by T-regulatory cells is IL-10. This cytokine directly and indirectly suppresses the activity of mast cells, basophils and eosinophils (Taams et al., 2006). IL-10 also prevents immunoglobulin heavy class switching from IgG4 to IgE (Akdis, 2008; Meiler et al., 2008). These functions are also carried out by the cytokine TGF-β, which is also secreted by Treg cells. TGFβ interacts with fibroblasts and myofibroblasts and is known to reduce peribronchiolar extracellular matrix deposition and airway smooth muscle cell proliferation. Mast cell degranulation is also suppressed by the interaction of OX40 on the surface of Tregs with OX40 ligand (OX40L) on mast cells (Gri et al., 2008).

Treg cells have also been shown to prevent the secretion of IL-9 and IL-3 by Th2 cells, thereby preventing mucus production in the lung. They also inhibit the function of the Th1 CD4+T cells and the production of Th1 cytokines in addition to suppressing CD8+ T cell activity (Romagnani, 2006). The suppressive effect of the Tregs on these various immune cells consequently results in reduced allergic pathology.

Treg cells in the control of autoimmune pathology

Studies utilizing a number of different experimental models of organ-specific autoimmune disease provide convincing evidence that specialized Treg cells capable of

controlling autoimmunity are an integral part of the T- cell repertoire in normal individuals. These models have common characteristics: they usually involve manipulation of lymphocyte homeostasis, particularly influencing the thymus or peripheral T cells, and autoimmunity can be inhibited by adoptive transfer of CD4+ T cells from normal animals (Shevach, 2001; Sakaguchi, 2000). The CD4+CD8- population of thymocytes is a potent source of Treg cells that adoptively transfer protection from autoimmune diabetes in rodent models (Saoudi et al., 1996). Autoimmune disease can be induced by reconstituting immunodeficient nude mice with CD4+ T cells from normal adult mice that have been depleted of CD4+ CD25+ T cells (Sakaguchi et al., 1995). CD4+ CD25+ T cells prevent the development of autoimmunity induced by their CD4+CD25- counterparts, which confirms that Treg cells are predominantly present in the CD4+CD25+ T cell population (Asano et al., 1996).

T regulatory cells in infectious disease

It has been suggested that several types of regulatory cells may exist, including Treg cells that are induced in response to infectious challenge (Bluestone and Abbas, 2003; Piccirillo and Shevach, 2004). Treg cells have been shown to respond to a large variety of self antigens, and growing evidence suggests that these cells may also respond to antigens expressed by microbes. Natural Treg cells appear to be associated most often with chronic infections. The influence of natural Treg cells may favorably affect the outcome of infection, or they may be harmful to the host (Belkaid and Tarbell, 2009). However, it is important to note that the outcome of infection is also affected by a variety of other factors. These factors include the stage of infection, dose of the pathogen, genotype and immunological status of the host, as well as the presence of concomitant disease or other infections (Belkaid and Rouse, 2005).

Some of the earliest studies of natural Treg cells emphasized that such cells help control the extent of immune-mediated pathology. In fact, a chief function of natural Treg cells may be to respond to signals associated with tissue destruction. It has been suggested that Treg cells may function to minimize collateral tissue damage triggered by inflammation (Powrie et al., 2003). Treg cells have been shown to act as chief regulators of intestinal lesion formation in mouse models of colitis. Generally, targets of natural Treg cells could include components of the innate immune system as well as T cells capable of causing immune pathology (Maloy, 2003).

Treg cells have also been shown to enhance pathogen survival and, in some cases, even support the long-term persistence of the pathogen. The mouse model of *L. major* infection provides a good example of this phenomenon. Natural Treg cells accumulate at the site of infection by *L. major* and limit the efficacy of Th1 immune responses (by both IL-10-dependent and IL-10-independent pathways) (Mendez et a, 2004). As a consequence, the natural Treg cells promote pathogen persistence and potential transmission to other hosts. Removal of natural Treg cells leads to “sterile cure”, a state that is not compatible with the preservation of long-term immunity (Belkaid and Rouse, 2005; Belkaid and Tarbell, 2009).

A number of investigators have suggested that other pathogens may be able to increase their survival by exploiting the Treg cell population in their host (Ge et al., 2008. However, recent evidence suggests that monoclonal antibody depletion of Treg cells from mice prior to and during challenge with a lethal strain of the protozoan parasite *Trypanosoma cruzi*, neither improved nor worsened the outcome of infection (Kotner and Tarleton, 2007). A similar study published in 2008 (Sales et al., 2008) provided evidence for only slightly enhanced resistance to infection with the Colombian strain of *T. cruzi* (lower parasitemia and mortality) following in vivo depletion of

CD4+CD25+ T cells. The authors concluded that CD4+CD25+ cells play only a limited role in host resistance during early *T. cruzi* infection (Sales et al., 2008).

The primary goal of the present study was to determine the impact of in vivo depletion of CD4+CD25+ Treg cells in a murine model of infection with the protozoan parasite, *T. gondii*. Specific aims of the study were as follows: 1) to successfully grow the 7D4 hybridoma cell line in culture and isolate sufficient quantities of anti-CD25 monoclonal antibodies for depletion studies, 2) to optimize an immune-fluorescent protocol for evaluating the success of *in vivo* depletion of CD25+ T lymphocytes, and 3) to evaluate the impact of CD25+ T-cell depletion on mortality, weight, and tissue cyst burdens in mice infected with the ME49 strain of *T. gondii*.

MATERIALS AND METHODS

Mice

Female Swiss Webster mice were obtained from Hilltop Laboratories (Scottsdale, PA), and C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor. ME). C57BL/6J mice were used in one preliminary experiment, Swiss Webster mice were used for all other experiments. C57BL/6J mice are highly susceptible to the ME49 strain of *T. gondii*, and typically develop severe encephalitis during the course of infection. Swiss Webster mice are more resistant to the ME49 strain and are commonly used as a model of *T. gondii* infection with the expected resolution of the acute phase of infection and development of tissue cysts in the chronic phase. All mice were 6 to 7 weeks of age at the initiation of each experiment. In addition, the mice were housed in plastic box cages (5 mice per cage) in a Super Mouse 750 cage system (Lab Products, Seaford, DE) and were provided rodent chow and water *ad libitum*.

Toxoplasma gondii infection

Tissue cysts of the ME49 strain of *T. gondii* were used to infect mice. Swiss Webster mice in the chronic stage of infection were killed by administering an overdose of the inhalant anesthesia, isoflurane. Cervical dislocation was then performed to ensure death. Brains were removed and disrupted using a Pyrex homogenizer and Teflon pestle. Tissue cysts number was determined by the method described by Huskinson-Mark et al. (1991). Two 15-µl aliquots of each sample of brain homogenate were counted using a hemacytometer. Each experimental mouse was infected by intraperitoneal (i.p.) injection

with 1000 tissue cysts (preliminary experiment) or 20 tissue cysts (all other experiments) diluted in sterile phosphate buffered saline (PBS).

In vivo depletion with anti-CD25 monoclonal antibody

A B cell hybridoma cell line (7D4; American Type Culture Collection) secreting antibody specific for the interleukin-2 (IL-2) receptor alpha chain was grown in protein-free hybridoma medium II (PFHM; Invitrogen Corporation, Carlsbad, CA). Culture supernatants were routinely harvested, spun at 400xg to remove cells and debris, filtered through a 0.22 µm filter, and stored for less than 2 months at 4°C. To precipitate the antibodies, an equal amount of ice-cold saturated ammonium sulfate solution was added to the hybridoma supernatant, mixed at 4°C with a stir bar for 24h, and then spun at 1,500xg in a centrifuge for 30 min at 4°C. Protein pellets were resuspended in PBS and then dialyzed until isotonic against PBS at a temperature of 4°C. Protein concentration was determined by the modified Bradford assay (Bradford, 1976). The antibody solution was then filter sterilized using a 0.22µm filter, and stored at- 80°C.

Mice were depleted of CD25 + T cells by intraperitoneal (i.p.) injection with 400µg of the anti-CD25 monoclonal antibody (Mab). Mice were injected once 7day prior to infection, and a second time 1 day prior to infection, with 20 tissue cysts of *T. gondii*. Previous investigators successfully depleted CD25+ T Cells using this Mab at concentrations ranging from 250µg to 1000µg (Kortner and Torleton, 2007). A direct immunofluorescence assy (IFA) was used to assess the degree of depletion of CD25+ T cells. An FITC-conjugated anti-CD25 Mab (Rat anti-Mouse CD25/IL-2RA-FITC; Southern Biotech, Pittsburg, PA) was used in the direct IFA performed on spleen cells isolated from representative mice from each treatment group on the day of infection.

Spleen cell preparation and immunofluorescence assay

Representative mice from each group were killed by administering an overdose of the inhalant anesthesia, isoflurane. Cervical dislocation was then performed to ensure death. Spleens were removed from each mouse and transferred to separate small Petri dishes containing 4-5 ml of PFHM. Splenocytes were dissociated from the capsule by repeated squeezing with long, curved forceps. The resulting cell suspension was then passed one time through a 22 gauge needle and one time through a 26 gauge needle to achieve a single cell suspension. The entire preparation of splenocytes was then spun at 1000 xg in a bench top centrifuge for 5 min. After decanting the supernatant, the pellet was resuspended in 500µl of PFHM. Erythrocytes were lysed by adding 500µl of D.I. water, and then isotonicity was restored in less than 10 sec by adding 500µl of 10X PBS. Five ml of PFHM was added to the suspension, and the tube was then spun at 1000 xg. The supernatant was decanted, and 4-5 ml of PFHM was used to resuspend the pellet.

A direct IFA was performed on splenocytes using a rat anti-mouse CD25/IL-2RA-FITC conjugated monoclonal antibody (Mab; Southern Biotech, Birmingham, AL). One ml of each splenocyte suspension was placed into a 1.5 ml centrifuge tube and tubes were spun by centrifugation at 4000xg for 4 to 5 min. The supernatant was discarded and the pellet was resuspended thoroughly in 250µl of FITC-conjugated Mab diluted 1/50 in PFHM. Splenocytes were incubated in the Mab solution for 1 hr at RT with occasional agitation of the tube. Cells were then washed 4 times with at least 1ml of PFHM by repeated centrifugation at 4000 xg for 4-5 min. Cells were fixed by resuspending the final cell pellet in 250µl of neutral buffered formalin for 1hr on ice. Cells were washed 4

times with 1ml of PFHM, and the final pellet was resuspended in 250µl of PFHM and stained cells were observed by fluorescence microscopy.

Experimental design

The role of CD25+ Treg cells in experimental toxoplasmosis was examined in one preliminary experiment and two follow-up experiments. In the first preliminary experiment, six C57BL/6J mice were divided randomly into two groups of three mice. Mice in the first group were injected with the anti-CD25 Mab once, 7 day prior to the infection , and second time one day prior to infection with 1000 tissue cysts of the ME49 strain of *T .gondii*. Mice in the second group were injected with PBS one week and one day prior to infection with 1000 tissue cysts of the ME49 strain of *T. gondii*. Treatment groups are shown in Table 1.

	Group 1	Group2
Number of mice	3	3
Anti-CD25 (400 µg)	+	_
T. gondii infection (1000 tissue cysts)	+	+

Table 1. Experimental design of preliminary experiment.

Two additional experiments were performed using female Swiss Webster mice and the ME49 strain of *T. gondii*. In the first trial, 15 female Swiss Webster mice (6-7 wks of age) were weighed upon arrival and randomly separated into 3 cages. Mice were numbered by ear punch, placed into cages, and provided with rodent chow (Purina rodent chow #5001, Richmond, IN) and water *ad libitum*. Treatment groups are shown in Table 2.

	Group1	Group2	Group3
Number of mice	5 Mice	5mice	5mice
Anti-CD25(400 µg)	+	+	_
T. gondii infection (20 tissuecysts)	+	_	+

Table 2. Experimental design for Experiment 1- Swiss Webster mice.

In the second experimental trial, 25 female Swiss Webster mice were weighed upon arrival and randomly separated into 5 cages (5 mice per cage). Mice were numbered by ear punch, placed into cages, and were provided with rodent chow and water *ad libitum*. Treatment groups are shown in the Table 3.

	Group1	Group2	Group3
Number of mice	10	5	10
Anti-CD25(400 µg)	+	+	_
T. gondii infection(20 tissuecysts)	+	_	+

Table 3. Experimental design for Experiment 2- Swiss Webster mice.

Mouse Weight and Mortality

All mice were weighed upon arrival, and weekly throughout the course of each experiment. In addition, each animal was carefully examined every one to two days to assess morbidity and mortality.

Experimental tissue cyst number determination

The number of tissue cysts present in the brains of mice at six weeks post infection was determined by the technique described by Huskinson-Mark et al. (1991). Mice were killed by administering an overdose of the inhalant anesthesia, isoflurane, and cervical dislocation was then performed to ensure death. Brains were removed, placed in to 1ml 1X PBS, and disrupted using a Pyrex homogenizer and Teflon pestle. The resulting suspension was then passed 10 times through an 18-gauge needle and 10 times through a 22-gauge needle. Two 15-µl aliquots of each sample of brain homogenate were then counted using a hemacytometer. Alternatively, in the second experimental trial, brains were removed and cut in half along the mid-line. One half of the brain was placed into 4% glutaraldehyde for later histological analysis, and the other half of the brain was used to quantify tissue cysts according to the method described above.

Statistical Evaluation

Data were analyzed using SYSTAT. The program ANOVA (Analysis of Variance) and a two sample T test were used to test the effect of Treg cell depletion on mice infected with *T. gondii.* Dependent variables in the ANOVA included weight and number of tissue cysts. The number of tissue cysts refers to the mean number of tissue cysts present in the brains of mice after six weeks of infection. Weight refers to the percent weight change over the course of the experiment. Tukey's HSD Post Hoc method was used for multiple comparisons of means of % weight change. A Two sample T-test was used to compare the means of the number of the tissue cysts formed after 6 weeks of post infection.

RESULTS

Effect of Treg cell depletion on mouse weight

Experiment 1

Figure 1 shows the effect of Treg cells depletion on the average percent weight change in Swiss Webster mice from two weeks prior to infection to 6 weeks post infection. Mice that were Mab-treated and infected showed the greatest average per cent weight loss following infection. The per cent weight loss observed for mice in this group was nearly twice that observed for mice in the PBS-treated group (Group 1: -8.855%, Group 3: -4.103%). However, this difference was not statistically significant (P□0.05). Mice in the control group Mab treated but non-infected showed an average percent weight gain of 28.6% over the course of the experiment. Turkey's post hoc test showed significant differences in weight change between Group 1 & Group 2 and between Group 2 & 3.

Experiment 2

Figure 2 shows the effect of Treg cell depletion on the average percent weight change in Swiss Webster mice from two weeks prior to infection to 6 weeks post infection. Mice that were Mab-treated and infected and mice that were PBS treated and infected showed a nearly identical average percent weight loss over the course of infection (Group 1: -23.016%, Group 3: -23.50%). However, this difference was not statistically significant (P□0.050. Mice in the control group (Mab-treated but non-infected) showed a 16.6% average weight gain during the course of the experiment. Turkey's post hoc test showed significant differences in weight change between Group 1 & Group 2 and between Group 2 & 3.

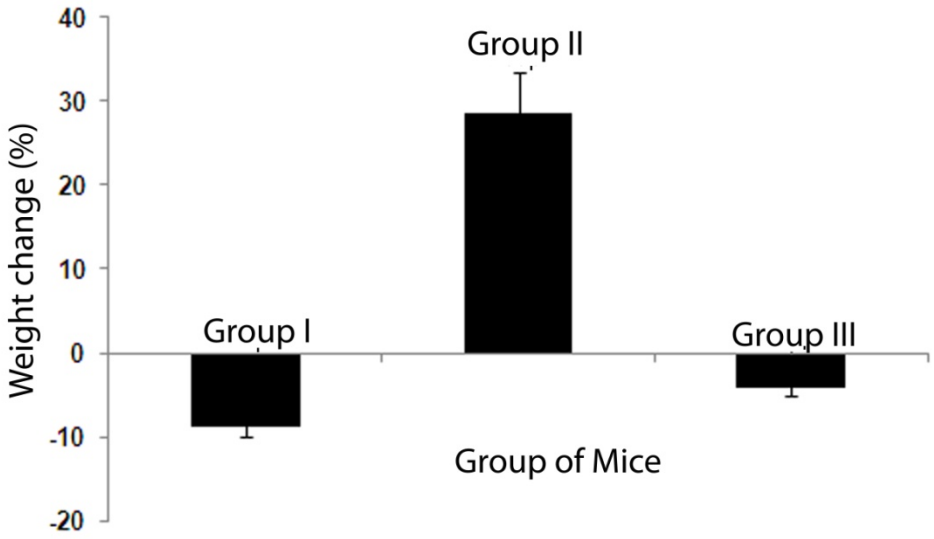

Figure 1. Effect of Treg cell depletion on weights of Swiss Webster mice in experiment 1. The average per cent weight loss of mice in each group (over a period of 6 weeks post-infection with *T. gondii*) was calculated. Values are means ± SE (*n*=5). ANOVA program was used to compare the % weight change for each of the three groups. The analysis assumed that the 3 samples from normal population with equal variance (p-value= 0.441). Tukey's HSD Post Hoc method was used for multiple comparisons of means of % weight change. By the results of ANOVA and multiple comparisons (using Tukey's method) there is no significant difference between Groups 1 and 3. However, there are significant differences between Groups 1 and 2 and between Groups 2 and 3(p-value<0.05).

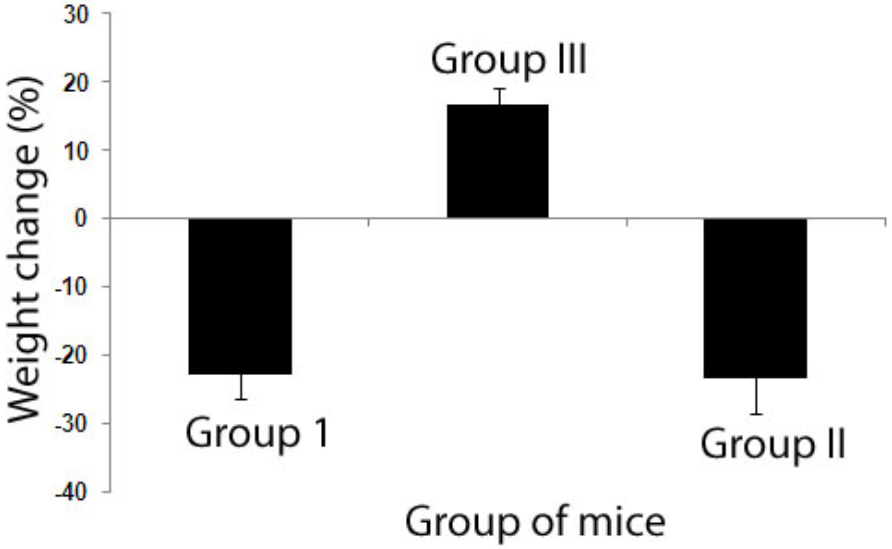

Figure 2. Effect of Treg cell depletion on weights of Swiss Webster mice in experiment 2. The average per cent weight loss of mice in each group (over a period of 6 weeks post - infection with *T. gondii*) was calculated. Values are means ± SE (*n*=10). ANOVA program was used to compare the % weight change for each of the three groups.The analysis assumed that the 3 samples from normal population with equal variance. F-test was used to compare the variability of the 3 populations.Tukey's HSD Post Hoc method was used for multiple comparisons of means of per cent weight change.Accordig to the results of ANOVA and multiple comparisons (using Tukey's method) there is no significant difference between Groups 1 and 3(P-value=0.192). However, there are significant differences between Groups 1 and 2 and between Groups 2 and 3 (P-value<0.05).

Effect of Treg cell depletion on tissue cyst number

The effect of Treg cell depletion on the number of tissue cysts was determined for all *T. gondii* -infected Swiss Webster mice. Table 4 & 5 shows the average number of tissue cysts in the brains of Swiss Webster mice inoculated i.p. with 20 tissue cysts of the ME-49 strain of *T. gondii* in experiment 1 and 2 respectively. Tissue cysts were counted six weeks post-infection.

A student T test showed no statistically significant difference (P□0.05) in the average number of tissue cysts present in the brains of mice in Group 1 (Mab-treated and infected) and Group 3 (PBS treated and infected) in both experiment 1 and 2.

Groups	Average No. Of Cysts	Standard Deviation
1 (Mab+ Infected)	380	±147
3 (PBS+Infected)	238	±106

Table 4: Number of tissue cysts formed in *Toxoplasma gondii*-infected C57BL/6J mice in experiment No 1. Mice in Group 1 were anti-CD25 Mab-treated and infected with 1000 tissue cysts. Mice in Group 3 were PBS treated and infected with 1000 tissue cysts. Data are presented as the average number of tissue cysts present in 1000µl (Total brain homogenate). By the results of Two-sample T test with equal population variances, we cannot conclude a difference in means of Groups 1 and 3(p-value= 0.136, with Degrees of Freedom (DF= 8).

GROUPS	AVERAGE NO. OF CYSTS	Standard Deviation
1 (Mab+Infected)	350	±213
3 (PBS+Infected)	540	±115

Table 5: Number of tissue cysts formed in *Toxoplasma gondii*-infected Swiss Webster mice in experiment No 2. Mice in Group 1 were anti-CD25 M ab treated and infected with 20 tissue cysts. Mice in Group 3 were PBS treated and infected with 20 tissue cysts. Data are presented as the average number of tissue cysts present in 1000µl (1/2 brain homogenate).By the results of Two-sample T test with equal population variances, we cannot conclude a difference in means of groups 1 and 3(p-value= 0.064, with Degrees of Freedom (DF= 14).

Immuno-fluorescent assay assessment of Treg cell depletion

The percentage of CD25+ T lymphocytes present in the spleens of Swiss Webster mice following Mab-treatment or PBS-treatment was determined by a direct immuno-fluorescence assay. Spleens from one PBS-treated mouse and one Mab-treated mouse were removed and a single cell suspension of splenocytes was prepared as described in Materials & Methods. Cells were stained by direct immuno-fluorescence utilizing a rat anti-mouse CD25/IL-2RA-FITC conjugated monoclonal antibody. Figure 3 shows the Immuno-fluorescent assay results for experiment 1.

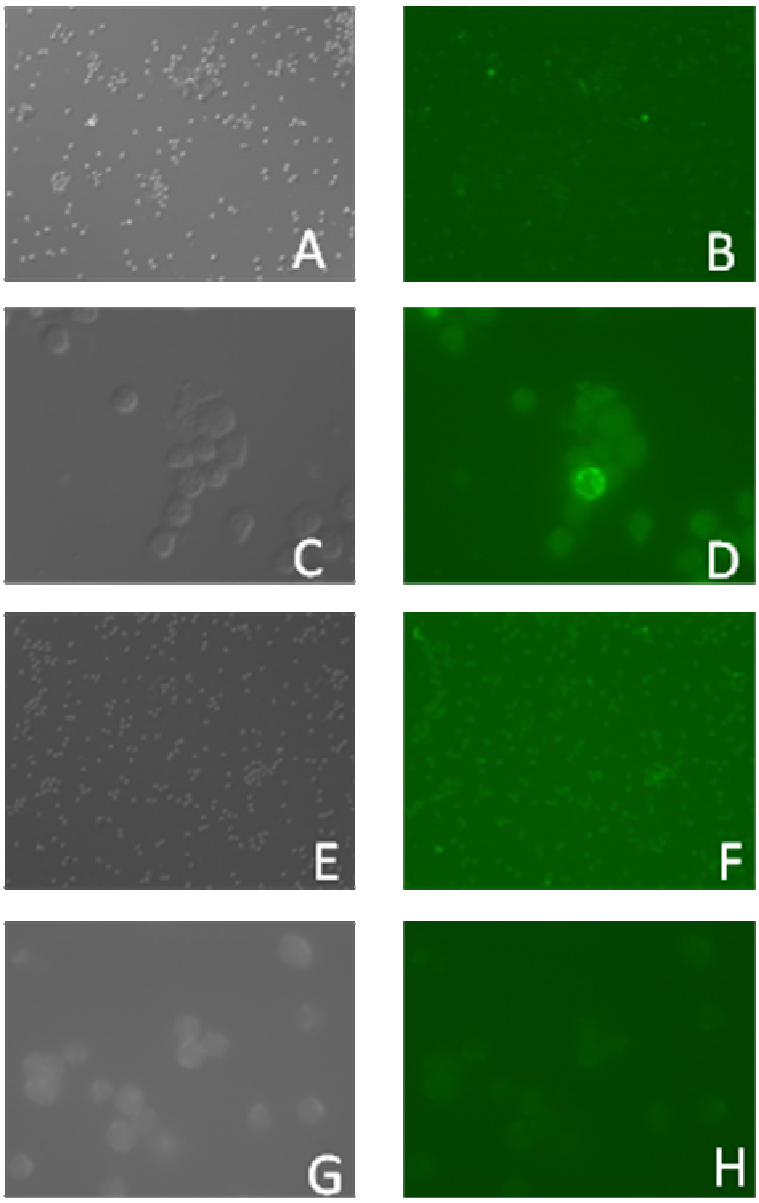

Figure 3. **Immuno-fluorescent assay to assess Treg cell depletion in experiment # 1.** Panel A- Differential interference contrast (DIC) image at 200 x magnification splenocytes from PBS treated mice. Panel B-FITC image at 200x magnification splenocytes from PBS treated mice. Panel C & D represents DIC, FITC images at 1000x magnifications of PBS treated mice respectively. Panel E - Differential interference contrast (DIC) image at 200 x magnification of splenocytes from Mab treated mice. Panel F- FITC image at 200 x magnifications of splenocytes from Mab treated mice. Panel G & H represents Mab treated DIC, FITC images of Mab treated mice at 1000x magnification. Approximately 200 cells were counted, about 10/200 cells showed fluorescence (panels A and B), where no fluorescence could be detected in any of the 200 cells in panels E and F.

Treg cell depletion in experiment 2:

A direct immuno-flourescence assay was performed to assess the extent of Treg cell depletion. Cells were stained by direct immuno-fluorescence utilizing a rat anti-mouse CD25/IL-2RA-FITC conjugated monoclonal antibody. Figure 4 shows the Immuno-fluorescent assay results of PBS treated and Mab treated mouse in experiment 2.

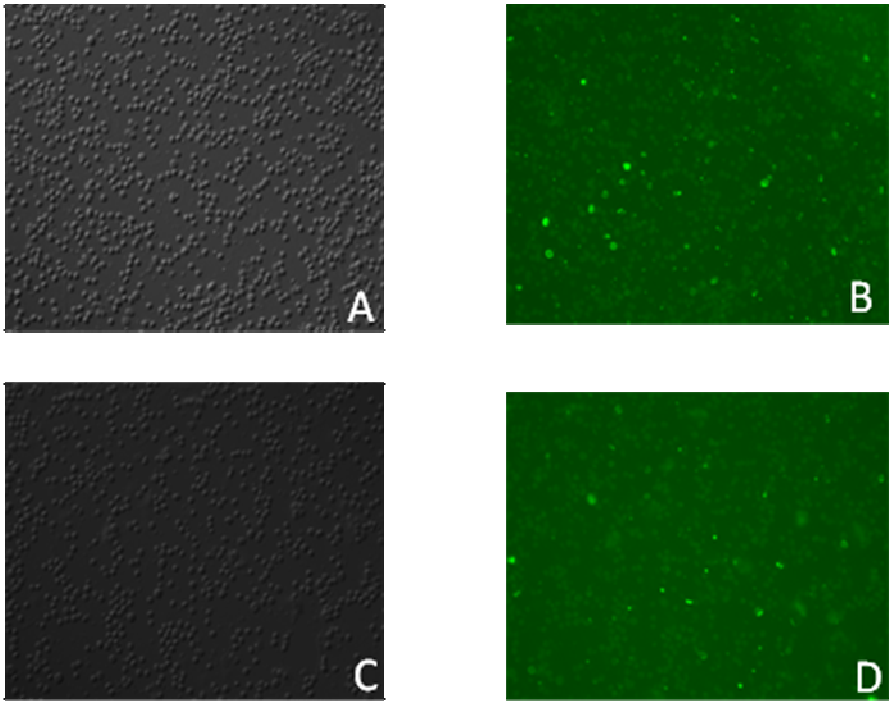

Figure 4. **Immuno-fluorescent assay to assess Treg cell depletion in experiment # 2.** Panel A- Differential interference contrast (DIC) image at 200 x magnification of splenocytes from PBS treated mice. Panel B- FITC image at 200x magnification of splenocytes from PBS treated mice. Panel C & D represents DIC, FITC images at 200x magnifications of Mab treated Mice. Approximately 200 cells were counted, about 12/200 cells showed Fluorescence in panels A and B, and 4/200 cells showed fluorescence in panels C and D.

Effect of Treg cell depletion on mortality rates of mice infected with *T. gondii*

C57BL/6J mice are highly susceptible to the ME49 strain of *T. gondii*, and typically show higher mortality rates than the more resistant Swiss Webster mice. A preliminary experiment was performed with a total of 6 C57BL/6J mice. Three mice were treated with anti-CD25 Mab and 3 were treated with PBS one week and one day prior to infection with 1000 tissue cysts of *T. gondii*. Mice in both treatment groups showed 100% mortality prior to the end of the second week of infection.

Mortality rates of Swiss Webster mice differed between experiments 1 and 2. In experiment 1, no mortality was observed over the six weeks of infection (data not shown). In contrast, in experiment 2, a 40% mortality rate occurred in group 3 (PBS treated and infected); whereas no mortality was observed in groups 1 or 2. Figure 5 shows the effect of anti-CD25 Mab-Treatment on survival of T. gondii infected mice in experiment 2.

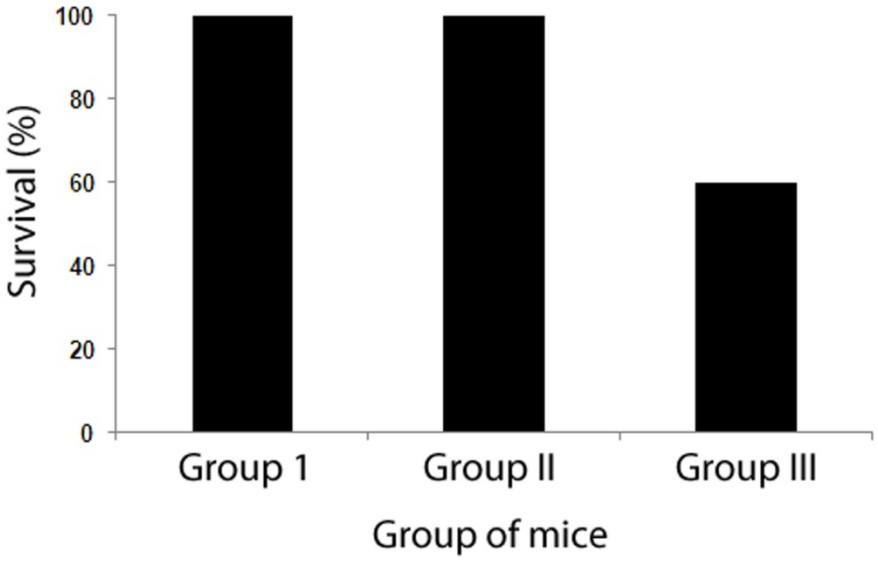

Figure 5. Effect of anti-CD25 Mab-treatment on survival of *Toxoplasma gondii*-infected mice in experiment 2. Swiss Webster mice were injected with 400 µg anti-CD25 Mab or PBS one week and one day prior to infection with *T. gondii*. Percent survival for each group (10 mice per group) is shown.

DISCUSSION

T regulatory cells play an important role in our immune system in regulating the activity of other T lymphocytes. The Treg cells possess specialized membrane glycoproteins known as CD4 and CD25, and express the transcription factor known as Foxp3. These CD4+ CD25+ Foxp3+ Treg cells comprise approximately 5 to 10% of total peripheral CD4+ T cells (Kotner and Tarleton, 2007). These cells are known to play a key role in the regulation of the immune response to tumors and infectious agents and also provide protection against autoimmune diseases. Many studies have provided evidence for the suppressive effects of Treg lymphocytes against effector CD4+ Tcells (Belkaid, 2003, Kotner and Tarleton, 2007; and Couper et al., 2009) In addition, the results of a variety of recent studies have provided evidence that Treg cells also have the ability to suppress cytokine production and proliferation of CD8+ T lymphocytes (Belkaid and Tarbell, 2009). These findings have led several investigators to suggest that the immunosuppressive function of Treg cells makes them a potential target for manipulation by some parasites for host immune evasion (Belkaid and Rouse, 2005; Kotner and Tarleton, 2007).

Several studies have been conducted to investigate the antigen specificity and response of natural Treg cells during infection with *Leishmania*. Parasite-specific Treg cells have been shown to accumulate at the site of infection during chronic *Leishmania* infection in mice, and have been found to proliferate strongly in response to infected dendritic cells in vitro (Suffia et al., 2006). In addition, Foxp3 mRNA expression levels have been measured in tissues from patients with Acute Cutaneous Leishmaniasis (ACL) and Chronic Cutaneous Leishmaniasis (CCL) (Bourreau et al., 2009).

These investigators demonstrated that Treg cells preferentially accumulate in lesions from ACL patients during the early phase of infection. In addition, levels of Foxp3 mRNA transcripts were significantly higher in specimens from patients with CCL than in those from patients with ACL suggesting a critical role of intralesional Treg cells. These intralesional Treg cells from both the ACL and CCL patients were shown to have suppressive functions in vitro since they inhibited IFN-γ production by CD4+CD25+ T cells (Bourreau et al., 2009). In contrast, CD4+CD25+ cells have been shown to play only a limited role in host resistance in murine models of infection with the protozoan parasite, *T. cruzi* (Kotner and Tarleton, 2007; Sales et al., 2008).

The purpose of the present study was to evaluate the role of Treg lymphocytes in a mouse model of infection with the protozoan parasite, *Toxoplasma gondii*. In this study, the impact of CD25+ cell depletion prior to infection was investigated in both C57BL/6 and Swiss Webster mice. C57BL/6J mice are highly susceptible to the ME49 strain of *T. gondii*, whereas Swiss Webster mice are more resistant and are commonly used as a model of *T. gondii*-infection with the expected resolution of the acute phase of infection. The effect of CD25+ cell depletion on the course of infection was evaluated by monitoring weight, tissue cysts numbers, and mortality.

In a small-scale preliminary experiment, C57Bl6 mice were depleted of Treg cells using an anti-CD25 Mab, or were sham injected with PBS. In vivo injection of anti-IL-2 receptor alpha-chain- specific Mabs has been shown to deplete Treg cells and abrogate their suppressive effects (Belkaid and Tarbell, 2009). Mice were then infected with 1000 tissue cysts of *T. gondii*, and were observed daily for signs of morbidity and mortality. A mortality rate of 100% was observed in both groups of infected mice prior to the third

week of infection. In this highly susceptible mouse model, using relatively high numbers of tissue cysts, the depletion of CD25+ cells prior to infection appeared to have negligible impact on the outcome of infection. For subsequent experiments, we used Swiss Webster mice, which are more resistant to the ME49 strain of *T. gondii*. We also used a lower infectious dose of 20 tissue cysts per mouse in attempt to avoid high rates of mortality during the acute phase of infection.

In the two experiments utilizing Swiss Webster mice, we evaluated the effect of Treg cell depletion on mice by recording weight changes, mortality rates, and number of tissue cysts present in mouse brains at six weeks post-infection. Over the course of the first experiment we observed weight changes in all three groups of mice. Mice in group 1, which were Mab-treated and infected with *T. gondii,* showed a greater average % weight loss following infection than mice in group 3 (PBS-treated and infected). However, these apparent differences were not statistically significant. As expected, mice in the control Mab-treated group (group 2) showed an increase in weight over the course of the experiment. This result demonstrates that the Mab treatment alone did not have a detrimental effect in control, non-infected mice.

The effect of Treg cell depletion on tissue cyst number was also determined in experiment 1. The number of tissue cysts present in the brains of infected mice was determined at 6 weeks post-infection. Mice in group 1 (Mab-treated and infected) showed a greater average number of tissue cysts as compared to mice in group 3 which were PBS-treated and infected. However, differences in average tissue cyst number between mice in group 1 and mice in group 2 were not statistically significant.

In a repeat experiment utilizing Swiss Webster mice, the sample size for each group was increased to 10. In this experiment, mice in groups 1 and 3 showed a very similar average % weight loss over the course of the experiment. In addition, there was no significant difference in tissue cyst number between Mab-treated and PBS-treated mice following six weeks of infection with *T. gondii*.

The impact of Treg cell depletion on mortality was also monitored throughout the course of both experiments. In experiment 1, no mortality was observed up to the termination of the experiment at six weeks post-infection. In contrast, in experiment #2 there was a significant increase in mortality observed for mice in group 3 (PBS-treated and *T. gondii*-infected). A total of 4/10 mice in group 3 (PBS-treated and infected) died within the first 3 weeks of infection. No mortality was observed for mice in group 1 (Mab-treated and infected) or in group 2 (Mab-treated and non-infected).

Near the conclusion of the present study, and during the preparation of this paper, Couper et al. (2009) published a very similar study designed to investigate the role of Treg cells during murine infection with *T. gondii*. The investigators injected C57BL/6J mice with anti-CD25 monoclonal antibodies (Mabs) prior to and during different phases of infection with 10 tissue cysts of the ME49 strain of *T. gondii*. The investigators reported that anti-CD25 Mab-treated C57BL/6J mice showed significantly reduced weight loss and reduced liver pathology during early acute infection (1-2 weeks). Mice depleted of CD25+ T cells also showed significant reduction in IFN-γ production during the early stages of infection, suggesting that effector CD4+ T cell activity was also impaired by the anti-CD25 Mab treatment. The CD25-depleted mice showed higher mortality rates and elevated numbers of tissue cysts in the late acute and early chronic

stages of infection as compared to sham-injected control mice. Based upon these results, the authors concluded that administration of anti-CD25 Mab targets effector CD4+ T cells in addition to natural Treg cells which constitutively express CD25. Their findings clearly illustrate the important limitations in the use of anti-CD25 Mabs to examine the function of Treg cells in experimental models of infectious disease.

Monoclonal antibodies specific for CD25 have also been used to investigate the role of natural Treg cells during murine infection with the protozoan parasite, *Trypanosoma cruzi* (Kotner and Tarleton, 2007; Sales et al., 2008) Kortner and Tarleton (2007) reported that parasitemia, host survival, and *T. cruzi* antigen-specific immune responses were not significantly different in CD25-depleted and non-depleted control mice. Furthermore, Treg cells were not observed in the muscle tissues of chronically infected mice and depletion of Treg cells during chronic infection had no therapeutic effect. Therefore, the authors concluded that CD4+ CD25+ Treg cells are not required for *T. cruzi* evasion of host immune responses. In addition, the authors concluded that Treg cells are also not responsible for CD8+ Tcell dysfunction in sites of parasite persistence (Kotner and Tarleton, 2007).

In the present study, we used an anti-CD25 Mab, isolated from the 7D4 hybridoma cell line in an attempt to deplete CD25+ cells in mice prior to infection. Using this approach, we were successful in achieving >95% depletion of CD25+ spleen cells prior to infection in experiment 1. However, using the same protocol in experiment #2, we were successful in reducing the percentage of CD25+ cells by only 33%. Depletion results achieved by other investigators using the same or similar Mabs are typically >90% (Kotner and Tarleton, 2007; Couper et al., 2009). The failure to achieve high levels of

CD25+ T cell depletion in our second experimental trial, most likely led to the differences we observed in trends in average tissue cyst burden, weight loss, and mortality between the two experiments. It has been reported that the CD4+ CD25+ T cell population regenerates quickly, and it may be possible to achieve more reliable levels of depletion using additional injections of the Mab, and/or using a higher concentration of Mab for each injection. A second important consideration is that the anti-CD25 Mab depletes not only CD4+CD25+ Treg cells, but also activated effector T cells and other cells that express CD25 (Couper et al, 2009). In future studies, the use of Foxp3 gene knockout mice or Foxp3 gene silencing techniques may be a better experimental approach for investigating the role of natural Treg cells during infection with *T. gondii* or other pathogens.

Finally, the results of the present study suggest that depletion of CD25+ cells has little measurable impact during the acute stage of infection with the ME49 strain of *T. gondii*. Even in experiment 1, when nearly 100% depletion of splenic CD25+ T cells was achieved, we observed no statistically significant differences in tissue cyst number, weight loss, or mortality between CD25 depleted and non-depleted mice infected with *T. gondii*. However, further studies will be required to fully investigate the role of Treg cells during the acute phase of infection with *T. gondii*, and to determine what role, if any, these cells might play during the chronic stage of murine toxoplasmosis.

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